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# Antiviral Activity of Broad-Spectrum and Enterovirus-Specific Inhibitors against Clinical Isolates of Enterovirus D68

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**We investigated the susceptibility of 10 enterovirus D68 (EV-D68) isolates (belonging to clusters A, B, and C) to (entero)virus inhibitors with different mechanisms of action. The 3C-protease inhibitors proved to be more efficient than enviroxime and pleconaril, which in turn were more effective than vapendavir and pirodavir. Favipiravir proved to be a weak inhibitor. Resistance to pleconaril maps to V69A in the VP1 protein, and resistance to rupintrivir maps to V104I in the 3C protease. A structural explanation of why both substitutions may cause resistance is provided.**

Human enterovirus D68 (EV-D68), first identified in 1962 in the United States, is a single-stranded, positive-sense RNA virus that belongs to the enterovirus genus in the family *Picornaviridae*. Until 2008, EV-D68 infections were only rarely detected and typically resulted in a mild respiratory illness (similar to a cold), with symptoms such as rhinitis, sneezing, coughing, and mild fever (1). From 2008 on, EV-D68 outbreaks were observed more frequently worldwide, including outbreaks in the Philippines in 2008 and in The Netherlands and Japan in 2010 (1–3). In 2014, EV-D68 caused the largest outbreak until now in the United States, with 1,153 people diagnosed with respiratory illness caused by EV-D68 (4). Those affected were mostly children with asthma or a history of wheezing. Occasionally, the virus has also been associated with polio-like illness, such as acute flaccid paralysis (AFP) and limb weakness (5, 6). Cases of severe AFP associated with EV-D68 infection were also reported in Europe (7), but a causal relationship was not established; EV-D68 was only detected in respiratory specimens during the episode of respiratory illness preceding AFP and not in central nervous system fluid.

So far, no vaccines or antivirals are available for the prophylaxis or treatment of EV-D68 infections. Hence, effective and safe drugs against EV-D68 infection are sought (8). Earlier studies revealed that the capsid-binding compound pleconaril and the 3C protease inhibitor NK-1.8k inhibit the replication of the EV-D68 Fermon strain (9, 10). The latter compound is a peptide aldehyde with few druglike properties. Some of these published results are conflicting (see below); moreover, only one or a few strains or isolates were used in these studies, and they did not represent the three different genogroups (8, 9). We therefore carried out a comparative *in vitro* antiviral study of known enterovirus-specific and broad-spectrum inhibitors against a selection of EV-D68 strains of the three major clusters (A, B, and C). To this end, a panel of 10 clinical isolates originating from The Netherlands (RIVM, Bilthoven, The Netherlands), Thailand, or the United States during the 2014 outbreak (obtained from the Centers for Disease Control and Prevention, USA, via BEI Resources [www.beiresources.org]) were selected that consisted of representative strains of clusters A, B, and C (Table 1) (2). The received viruses were cultivated on HeLa Rh cells in the presence of 30 mM $MgCl_2$ at 35°C.

The following enterovirus and broad-spectrum inhibitors were included in this study: (i) the capsid binders (CBs) pleconaril, vapendavir, and pirodavir; (ii) the 3C protease inhibitors (PIs) rupintrivir and SG85 (11); (iii) the host cell-targeting molecule enviroxime; and (iv) the broad-spectrum antiviral favipiravir (T-705) (12, 13). The antiviral activity of the above-mentioned compounds against the panel of EV-D68 strains (multiplicity of infection, 0.001) was assessed in a cell-based cytopathic effect (CPE) reduction assay with a colorimetric readout using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium/phenazine methosulfate (MTS/PMS) method as described previously (14).

The PI rupintrivir proved to be an efficient inhibitor of the *in vitro* replication of all 10 EV-D68 isolates, with mean 50% effective concentrations ($EC_{50}$s) ranging from 0.0018 to 0.0030 μM (Table 1). Likewise, SG85, a Michael acceptor inhibitor of the EV-D68 3C protease, also efficiently inhibited all EV-D68 strains, with $EC_{50}$s ranging from 0.0022 to 0.0080 μM. Enviroxime, which targets the cellular phosphatidylinositol 4-kinase IIIβ (a kinase that is crucial for the replication of picornaviruses), inhibited the replication of all tested EV-D68 isolates, with $EC_{50}$s between 0.19 and 0.45 μM. Favipiravir, a drug that has been approved in Japan for the treatment of infections with influenza virus but that also exerts activity against other RNA

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**TABLE 1** Antiviral activity of selected compounds on the *in vitro* replication of EV-D68 strains

| Lineage | Strain | Year clinical specimen collected | $EC_{50}$ (μM)[a] with: | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | Pleconaril | Vapendavir | Pirodavir | Rupintrivir | SG85 | Enviroxime | Favipiravir |
| Cluster A | CU70[b] | 2011 | 0.13 ± 0.06 | 1.4 ± 0.5 | 2.4 ± 0.3 | 0.0027 ± 0.0005 | 0.0080 ± 0.0009 | 0.27 ± 0.04 | 97 ± 29 |
| | 4311000670[c] | 2010 | 0.28 ± 0.2 | 1.0 ± 0.1 | 3.8 ± 1.5 | 0.0027 ± 0.0005 | 0.0072 ± 0.0007 | 0.33 ± 0.09 | 88 ± 17 |
| | 4311000742[c] | 2010 | 0.14 ± 0.02 | 1.8 ± 0.8 | 7.1 ± 1.0 | 0.0026 ± 0.0008 | 0.0080 ± 0.003 | 0.45 ± 0.2 | 63 ± 27 |
| | 4310901348[c] | 2009 | 0.37 ± 0.2 | >50 | 9.6 ± 2.0 | 0.0030 ± 0.0003 | 0.0074 ± 0.0008 | 0.20 ± 0.07 | 101 ± 20 |
| | US/KY/14-18953[c] | 2014 | 0.16 ± 0.2 | 3.8 ± 0.9 | 5.5 ± 1.4 | 0.0024 ± 0.0003 | 0.0027 ± 0.001 | 0.43 ± 0.2 | 78 ± 7 |
| Cluster B | 4310900947[c] | 2009 | 0.42 ± 0.2 | 1.1 ± 0.3 | 2.7 ± 0.6 | 0.0029 ± 0.0002 | 0.0065 ± 0.003 | 0.20 ± 0.05 | 97 ± 12 |
| | 4310902042[c] | 2009 | 0.83 ± 0.9 | 1.1 ± 0.3 | 3.9 ± 3.0 | 0.0020 ± 0.0005 | 0.0051 ± 0.003 | 0.19 ± 0.04 | 121 ± 31 |
| | US/IL/14-18952[d] | 2014 | 0.26 ± 0.2 | 1.3 ± 0.7 | 4.7 ± 4.2 | 0.0018 ± 0.0005 | 0.0032 ± 0.001 | 0.20 ± 0.1 | >100 |
| | US/MO/14-18947[d] | 2014 | 0.39 ± 0.6 | 0.5 ± 0.3 | 3.1 ± 1.7 | 0.0019 ± 0.0007 | 0.0022 ± 0.0004 | 0.21 ± 0.07 | >100 |
| Cluster C | 4310902284[c] | 2009 | 0.081 ± 0.03 | 1.2 ± 0.1 | 2.7 ± 1.0 | 0.0028 ± 0.0002 | 0.0045 ± 0.002 | 0.19 ± 0.1 | 79 ± 14 |

[a] The antiviral activity of the compounds against 10 EV-D68 isolates was determined in HeLa Rh cells by a CPE reduction assay (multiplicity of infection, 0.001). Cell viability was measured with a colorimetric readout using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium/phenazine methosulfate (MTS/PMS) method. Data shown are means ± SD from at least three independent experiments.
[b] From Thailand (22).
[c] From The Netherlands (23).
[d] From the United States (24).

viruses, including Ebola virus, proved to be a weak inhibitor of the *in vitro* replication of EV-D68 ($EC_{50}$, ≥63 μM).

Conflicting data were reported on the antiviral activity of the capsid binder pleconaril against EV-D68. On the one hand, pleconaril was reported to be unable to inhibit the currently circulating EV-D68 strains (8). In contrast, another study showed good antiviral activity of pleconaril against the EV-D68 Fermon and U.S. 2014 strains (9, 15). Here, we observed that pleconaril was active against all 10 clinical EV-D68 strains from the selected reference panel, including EV-D68 strains that circulated in the United States in 2014. The observed $EC_{50}$s were comparable with the earlier reported $EC_{50}$s against the Fermon and U.S. 2014 strains (on HeLa H1 cells) (9, 15). Both vapendavir (Biota Pharmaceuticals), currently being studied in a phase 2 clinical trial of adults with moderate to severe asthma with symptomatic rhinovirus infection, and pirodavir inhibited EV-D68 replication. Vapendavir was on average 3-fold more active than pirodavir but proved inactive to one of the cluster A viruses (Table 1). However, for the U.S. strains, some residual replication was still observed, even at the highest concentration tested (50 μM, following microscopic inspection).

To determine whether pleconaril-resistant and rupintrivir-resistant EV-D68 variants can be selected, a clonal resistance selection protocol was performed, as described previously (16), using the CU70 strain. Briefly, a CPE reduction assay was compiled that consisted of a combination matrix of the compound concentration and virus input. Next, several 96-well plates with adherent HeLa Rh cells were set up in the presence of the optimal viral input and compound concentration (this is the highest viral input and lowest compound concentration at which full inhibition of virus-induced CPE can be observed). After 4 days of incubation at 35°C, supernatant was harvested only from wells with complete virus-induced CPE. The supernatant from a number of such cultures, which may carry compound-resistant virus variants, were titrated in the presence of the same compound concentration to enrich the compound-resistant virus variants. At 4 days postinfection, supernatant of virus-infected compound-treated cultures with the lowest viral input and for which 100% CPE was still observed was harvested. As a result of the above clonal selection methodology, resistant variants of EV-D68 emerged in 9 of 144 cultures (6%) that were treated with pleconaril, versus only 1 in 288 cultures (0.3%) treated with rupintrivir. This is comparable to our findings for human rhinovirus 14 (17). The antiviral phenotype of two of the putative pleconaril-resistant EV-D68 variants and the rupintrivir-resistant EV-D68 variant were subsequently characterized, revealing a >15-fold decrease in susceptibility to the antiviral effect of pleconaril ($EC_{50}$, 0.13 ± 0.06 μM *versus* 1.9 ± 0.05 and 4.1 ± 1.8 μM for the wild-type (WT) and two resistant variants, respectively) (Fig. 1A) and a 7-fold decrease in susceptibility to the antiviral effect of rupintrivir ($EC_{50}$, 0.0027 ± 0.0005 μM *versus* 0.020 ± 0.006) (Fig. 1B). A cross-resistance evaluation with pirodavir proved that this compound was >23-fold less active against the pleconaril-resistant variants, while SG85 was 5-fold less active against the rupintrivir-resistant EV-D68 variant than against WT EV-D68 ($EC_{50}$, 0.0080 ± 0.0009 μM *versus* 0.044 ± 0.005). The two pleconaril-resistant EV-D68 variants were found to share one amino acid substitution, i.e., V69A (according to the numbering in reference 9) in the VP1 protein; an additional K155E substitution was also detected in one of the pleconaril-resistant EV-D68 variants (this substitution was not reported earlier in pleconaril-resistant enteroviruses [18, 19]).

A modeling study was performed to elucidate the role of amino acid substitution V69A in the VP1 protein in antiviral resistance (Fig. 2A). This study revealed that one of the methyl groups on the benzene ring in pleconaril engages in Van der Waals contact with atom CG2 of residue V69 in chain A (4.3 Å), pointing to a possible explanation for the valine to alanine substitution effect that results in resistance. Substitution to alanine removes the CG1 and CG2 atoms cancelling any possible Van der Waals stabilizing contact with the inhibitor. Furthermore, the Val69 CG1 and CG2 atoms make hydrophobic contacts with L103 and M241 side chains, which stabilize the protein matrix. Substitution of the valine to alanine might disturb the assembly in the interior of the protein, which in turn

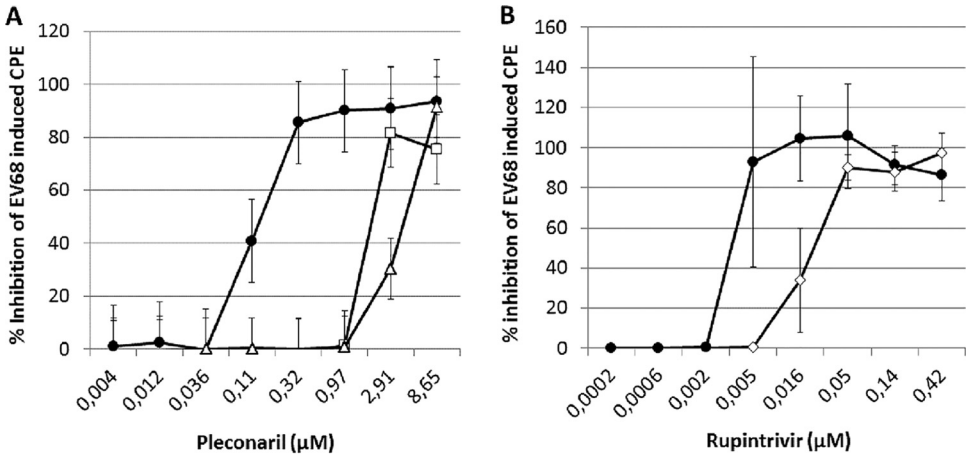


**FIG 1** Resistance of EV-D68 CU70 variants to compounds. (A) The antiviral activity of pleconaril against WT (black dot) and pleconaril-resistant variant 1 (V69A, white square) and variant 2 (V69A + K155E, white triangle) was determined in a CPE reduction assay with an MTS readout. (B) The antiviral activity of rupintrivir against WT (black dot) and a rupintrivir-resistant variant (V104I, white diamond) was determined in a CPE reduction assay with an MTS readout. Data shown are means ± SD from three experiments.

may result in conformational rearrangement with secondary effects on the binding of pleconaril.

The rupintrivir-resistant mutant was shown to carry a single amino acid substitution (V104I) in the 3C protease. We analyzed the steric consequences of this mutation on the basis of the crystal structure of the EV-D68 3C protease in complex with SG85 (11), which shares identical P1′ and P1 moieties with rupintrivir. The mutation site is >10 Å away from the inhibitor binding site. In fact, isoleucine is present at this position in all enteroviruses of groups A (such as EV-A71) and B (such as CVB3). Although the model seems to suggest that the V104I mutation might lead to a conformational rearrangement of the side chain of Q146 (Fig. 2B), the crystal structures of the 3C proteases of EV-A71 and CVA16 in complex with rupintrivir (20) show that this is not the case. Instead, the difference at position 104 may lead to different conformations of the loop 104 to 108, which in turn influences the exact position of the main-chain carbonyl of E24 (Fig. 2B) (20). Differences in affinities of rupintrivir to various 3C proteases have been ascribed, in part, to the different sizes of the S1′ site due to variable orientations of E24 (20). It is conceivable that the relatively small effects of the V104I mutation on the activity of rupintrivir against EV-D68 infection are afforded by a similar mechanism.

In conclusion, the antiviral activity of a panel of (entero)virus inhibitors against EV-D68 was evaluated using a reference panel of 10 clinical EV-D68 strains, representing the three major clusters. Both the capsid binders and the PIs inhibited viral replication, albeit with different potency. Pleconaril-resistant variants readily emerged, which was not the case with rupintrivir treat-

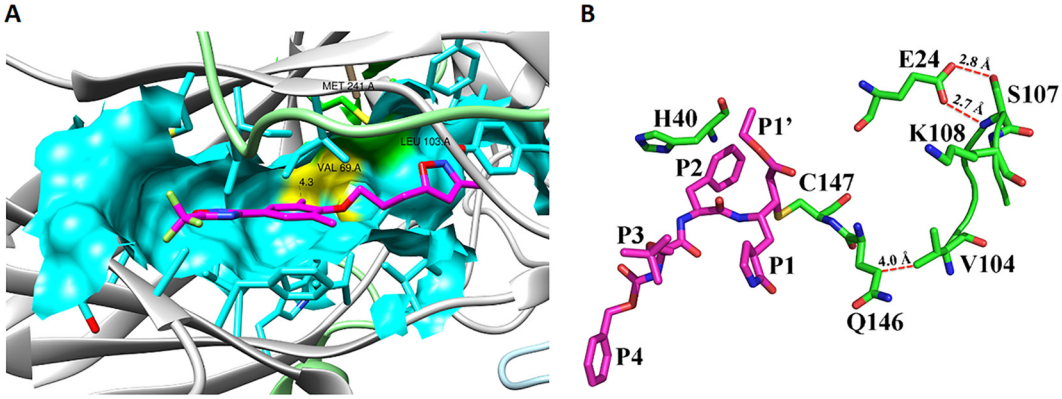


**FIG 2** (A) Modeling of pleconaril in the VP1 pocket of WT EV-D68. The 4WM7 Protein Data Bank (PDB) entry was used with the pleconaril structure. VP1 is shown as gray, VP2 as blue, VP3 as green, and VP4 as cyan ribbons. Pleconaril has magenta carbons. Interaction (mostly by hydrophobic contacts) residues are in cyan. The surface of the pocket is partly shown. Residue V69 is highlighted in yellow. M241 and L103, which make hydrophobic packing contacts with V69, are shown as a green surface. The image was created by UCSF Chimera (21). (B) The site of the rupintrivir resistance substitution in the crystal structure of the EV-D68 3C protease in complex with the inhibitor SG85 (PDB, 3ZVF) (11). Introduction of isoleucine at position 104 may lead to a conformational rearrangement of Q146. However, the crystal structures of EV71 and CVA16 3C proteases (where residue 104 is isoleucine) in complex with rupintrivir (21) show that this does not happen. Instead, the loop 104 to 108 may change conformation, leading to a reorientation of E24 and changes in the size of the S1′ pocket, thereby decreasing the affinity of the inhibitor. Carbon atoms of the protease are shown in green, and those of the inhibitor SG85 are shown in pink. The figure was prepared using PyMOL (Schrödinger).

ment. The data presented here offer a framework of reference data against which other inhibitors of EV-D68 replication can be compared.

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